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Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule

Richard P.Novick, Hope F.Ross, Steven J.Projan, John Kornblum, Barry Kreiswirth and Soraya Moghazeh

Department of Plasmid Biology, Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA

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The production of most toxins and other exoproteins in *Staphylococcus aureus* is controlled globally by a complex polycistronic regulatory locus, *agr*. Secretory proteins are up-regulated by *agr* whereas surface proteins are down-regulated. *agr* contains two divergent promoters, one of which directs the synthesis of a 514 nucleotide (nt) transcript, RNAIII. In this report, we show that the cloned RNAIII determinant restores both positive and negative regulatory functions of *agr* to an *agr*-null strain and that the RNA itself, rather than any protein, is the effector molecule. RNAIII acts primarily on the initiation of transcription and, secondarily in some cases, at the level of translation. In these cases, translation and transcription are regulated independently. RNAIII probably regulates translation directly by interacting with target gene transcripts and transcription indirectly by means of intermediary protein factors.

Key words: agr locus/*Staphylococcus aureus*/transcription regulation/translation regulation

Introduction

In *Staphylococcus aureus*, many exoproteins are synthesized and secreted at the end of exponential growth under the control of a global regulatory determinant known as *agr* (Recsei *et al.*, 1986). The *agr* response involves induction of the synthesis of many secreted proteins, including toxic shock syndrome toxin-1 (TSST-1), α-, β- and δ-hemolysins, and serine protease, and reciprocal repression of the synthesis of surface proteins, such as protein A, coagulase and probably fibronectin binding protein (Lindberg *et al.*, 1990). The secreted proteins are induced post-exponentially in agr^+ wild-type strains and are produced at very low levels or not at all in agr^- mutants. The surface proteins are produced throughout growth in both wild-type and mutant strains but at a much higher level in the mutants (Janzon *et al.*, 1986; Recsei *et al.*, 1986; Ross, 1989).

The centerpiece of the system is the complex polycistronic *agr* locus, located at about 4 o'clock on the standard *S.aureus* chromosomal map (Pattee *et al.*, 1990). The *agr* locus has been cloned and sequenced (Peng *et al.*, 1988; Janzon *et al.*, 1989) and found to consist of two divergent operons transcribed from promoters, referred to as P2 and P3, which are separated by ~120 bp. A diagram of the *agr* locus is presented in Figure 1A. Promoters P2 and P3 are temporally regulated, weakly active in early exponential phase and strongly active later in growth (Janzon *et al.*, 1989; Ross, 1989; Vandenesch *et al.*, 1991). A third promoter, P1, is located just 5′ to *agrA*. P1 is a weak constitutive promoter (Peng *et al.*, 1988) whose role in the system is unknown.

The P2 operon, 3 kb in length, contains four open reading frames, *agrA*, *agrB*, *agrC* and *agrD* (Kornblum *et al.*, 1990); the predicted product of *agrA* corresponds to the response regulators of the classical two component sensory transduction systems in bacteria (A.Ninfa, personal communication) and that of *agrB* to the histidine phosphokinase sensory transducers (Ross, 1989). Preliminary evidence suggests that the *agr* system may be activated by low pH (<6.9) (Regassa *et al.*, 1991). Strains defective in any one of the four ORFs of the P2 operon are agr^- and lack both P2 and P3 transcripts (Morfeldt *et al.*, 1988; Ross, 1989; Kornblum *et al.*, 1990), suggesting that the products of the four P2 ORFs function in concert to activate transcription from the two promoters; thus the regulatory circuit is autocatalytic. P3 initiates a 0.5 kb transcript, RNAIII, which encodes the *agr*-regulated protein, δ-hemolysin (Janzon *et al.*, 1989). Remarkably, an insertion in the RNAIII region, at the *Cla*I (1187) site (see Figure 1A), distal to the δ-hemolysin coding sequence (*hld*), inactivates *agr* function but does not affect the activity of the two promoters (Arvidson *et al.*, 1989). This result suggests that activation of the putative sensory transduction pathway encoded by the P2 operon is not *per se* sufficient to activate the *agr* response and suggests that the RNAIII region encodes the actual regulator.

We have previously shown that the cloned *agrA* determinant complements the pleiotropic exoprotein defect seen with *agrA* mutations such as *agrA316* (Tn*551*) (Recsei *et al.*, 1986) and that phenotypically similar mutations occurring elsewhere in the *agr* region are not complemented by the cloned *agrA* (Novick *et al.*, 1989a). One such mutation is *agr*-2, which has a 1 kb deletion encompassing P2 and P3 but not affecting *agrA* or its promoter, P1 (Kornblum *et al.*, 1990). Another is the above mentioned insertion in the RNAIII region (Arvidson *et al.*, 1989). These results suggested that the *agr* system contains two or more essential elements that act either independently or sequentially. Transcriptional analysis, showing that *agrA* mutations eliminate P2 and P3 activity (Arvidson *et al.*, 1989; Novick *et al.*, 1989a), suggested that *agrA* and a product of the P3 operon act sequentially: *agrA* activates P2 and P3 and a P3 product determines the exoprotein response.

In this report, we have verified the hypothesis that the RNAIII region encodes the *agr*-specific regulator by cloning the RNAIII determinant under control of the staphylococcal plasmid pI258 β-lactamase promoter (Novick and Richmond, 1965; McLaughlin *et al.*, 1981). Induction of this promoter activates the *agr* response in the absence of any other element of the *agr* system. Mutation and deletion analyses suggest that RNAIII itself, rather than any translation product, is the effector of exoprotein gene regulation. Subcloning data

indicate that RNAIII regulates exoprotein production primarily at the level of transcription but, in some cases, also at the level of translation.

Results

RNAIII activates the agr response

To test the hypothesis that the P3 operon encodes the *agr* effector, we subcloned three fragments, a27, a40 and a42 (see Figure 1) into the polylinker region of the P-*bla* vector, pRN6725. Inserts in this region are transcribed from the β-lactamase promoter carried by the vector. a27 and a40 contain the intact *rnaIII* determinant lacking its promoter, whereas a42 covers the P2 operon, also lacking its promoter. The resulting plasmids, pRN6735 (a27), pRN6848 (a40) and pRN6852 (a42), respectively, were transduced to the *agr*-null strain, RN6911, and tested for activation of the *agr* response. For most of the tests, the strains containing these clones harbored a second plasmid, pI524, which encodes the *bla* repressor. This was necessary because the high activity of the constitutive β-lactamase promoter causes any multicopy plasmid containing it to be unstable.

In Figure 2 are shown Western immunoblots of culture supernatants from strains containing these clones in the *agr*-null background. Note that the *agr*$^+$ strain, RN6390B, generates strong signals for β-hemolysin and α-hemolysin, whereas the *agr*-null strain, RN6911, generates a very weak β-hemolysin and no detectable α-hemolysin signal. As seen in the anti-β blot, for which a polyclonal anti-β-hemolysin serum was used, protein A is barely detectable in RN6390B

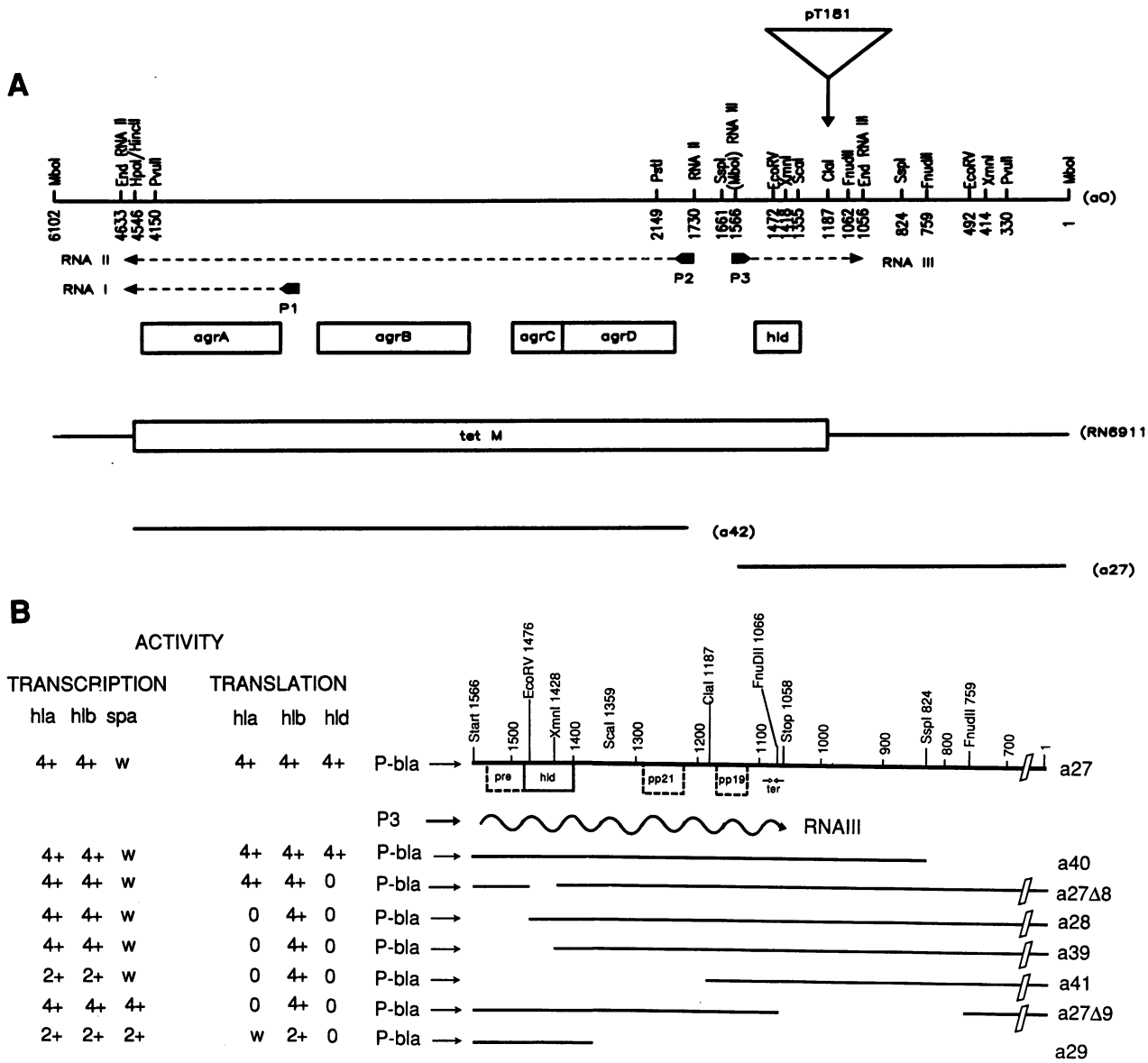

Fig. 1. (**A**) Organization of the *agr* locus. At the top is a restriction map of the 6.1 kb *agr*-a0 clone (Peng *et al.*, 1988) showing ORFs, promoters and transcripts (Kornblum *et al.*, 1990). Directly below is shown the corresponding region of the *agr*-null (RN6911) chromosome, in which the 3.4 kb *ClaI*(1187)–*HpaI*(4546) fragment has been replaced by the *tetM* marker (Nesin *et al.*, 1990), and above is shown the location of an inactivating insertion of pT181 described by Arvidson *et al.* (1989). (**B**) Organization of the RNAIII region showing subclones, deletions, and their *agr* activities. Under TRANSCRIPTION, figures represent the relative strength of signals on Northern blots, and under TRANSLATION, strength of signals on Western blots or activities on SBA; 'w' indicates very weak but detectable activity. Note that the sequence is broken between 1 and 700. *agr* subfragments, prefixed with 'a', are numbered in order of isolation.

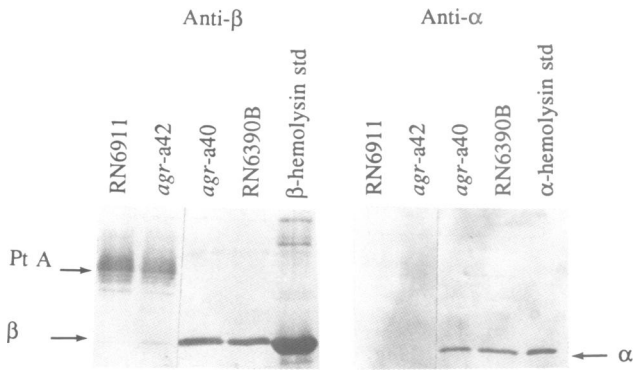

Fig. 2. Western immunoblot analysis of α-hemolysin, β-hemolysin and protein A. Control cultures (RN6911 and RN6390B) were grown to early stationary phase. Strains containing the indicated *agr* subclones were grown to a cell density of $1-1.5 \times 10^9$, then grown for an additional 2 h in the presence of an inducing concentration of CBAP (5 μg/ml). Culture supernatants were analyzed by SDS−PAGE electrophoresis followed by Western immunoblotting with the indicated antibody. All samples represent constant numbers of cells. The anti-β-hemolysin serum (kindly provided by Timothy Foster) was a rabbit polyclonal serum and the blot was developed with goat anti-rabbit alkaline phosphatase conjugate. The anti-α-hemolysin serum (kindly provided by Dr Sidney Harshman) was a mouse monoclonal and the blot was developed with goat anti-mouse alkaline phosphatase conjugate. Samples of purified β-hemolysin and α-hemolysin used as standards were kindly provided by Dr Pat Schlievert and Dr Sidney Harshman, respectively.

Table I. Effect of *agr* subclones on hemolysin production

	α-hemolysin[a]	β-hemolysin[a]
RN6911(*agr*$^-$)	<0.1	1.2
RN6390B(*agr*$^+$)	14.5	22.8
RN6911(a42)[b]	<0.1	4.0
RN6911(a41)	<0.1	22.0
RN6911(a29)	2.0	14.0
RN6911(a40)	15.7	28.6
RN6911(a39)	<0.1	4+[c]
RN6911(a28)	ND[d]	4+[c]

[a]Determined by densitometric scanning of Western blots. Data are in scanner units.
[b]Fragment numbers refer to plasmid subclones listed in Table II.
[c]Equivalent to RN6309B as seen on SBA plates.
[d]ND, none detected on SBA.

supernatants but is abundant in RN6911, consistent with the well-known reciprocal regulation by *agr* of the synthesis of these proteins. Protein A is not visualized in the anti-α panel because the anti-α-hemolysin serum is a mouse monoclonal which does not react with protein A. In the anti-β panel, it can be seen that the RNAIII clone, a40, induces β-hemolysin production to a level comparable with that seen with the *agr*$^+$ wild-type, RN6390B, whereas the cloned P2 operon (a42) increases β-hemolysin production very slightly, possibly not significantly above that seen with the *agr*-null strain. The strong protein A signal seen with RN6911 is undiminished by the a42 clone but reduced to the same low level by the a40 clone as that seen with the *agr*$^+$ wild-type, RN6390B.

In the anti-α panel, it can be seen that neither the *agr*-null strain (RN6911) nor the cloned P2 operon (a42) produces a detectable signal whereas the RNAIII (a40) clone and the *agr*$^+$ wild-type (RN6390B) produce strong, comparable α-hemolysin bands. These Western blots were evaluated quantitatively by densitometric scanning and the results for α-hemolysin and β-hemolysin are shown in Table I. The RNAIII stimulation of β-hemolysin was ~19-fold whereas RNAIII stimulation of α-hemolysin was at least 150-fold. These numbers are consistent with the fact that β-hemolysin is expressed at a detectable basal level in *agr*$^-$ strains whereas α-hemolysin is not. When cloned in the opposite orientation, neither the a27 nor the a40 fragment had any effect on the exoprotein activity of the *agr*-null strain (not shown).

The finding that the cloned RNAIII determinant can complement the *agr*-null mutation whereas the P2 operon cannot confirms the conclusion that the P2 genes and RNAIII act sequentially and supports the hypothesis that RNAIII is or encodes the *agr*-specific regulatory molecule. The orientation effect plus the inducibility of the RNAIII response together rule out the possibility that a *cis*-acting regulatory site is responsible for the activity of the RNAIII region.

The RNAIII molecule

The DNA sequence of the RNAIII region has been previously published (Janzon *et al.*, 1989) and the 5′ end of the RNAIII transcript determined by primer extension to be at position 1566 (see Figure 1). Using the S1 protection method (Berk and Sharp, 1977), we have confirmed the location of the 5′ end and have also determined the location of the 3′ end to be at nucleotide 1058 (not shown) within a run of T residues lying 3′ to a strong potential hairpin that presumably serves as a ρ-independent termination signal (see Figure 3).

We have predicted the secondary structure of RNAIII by means of the Zuker−Stiegler algorithm (Zuker and Stiegler, 1981) and this prediction is illustrated in Figure 3. Note that the molecule has a strong axis of symmetry centering at position 1330 (line) and that the predicted loop at that point closely resembles the loop of the 3′ hairpin which constitutes the RNAIII terminator. Note also that the 5′ and 3′ ends of the molecule are predicted to pair.

There are three potentially translatable reading frames, indicated in Figure 1B. The *hld* reading frame, which is known to be translated, is preceded by 18 in-frame codons (*pre-hld*) which are not known to be translated, nor are the other tB)wo, pp19 and pp21, respectively; *pre-hld* and pp21 each has a plausible Shine−Dalgarno site, whereas pp19 does not.

RNAIII acts at the level of transcription

In Figure 4 is shown a Northern blot analysis of RNA prepared from RN7220, a derivative of the *agr*-null strain containing the RNAIII clone a27 (pRN6735), and probed for *hlb*, *hla* and *spa* (protein A determinant) transcripts. As can be seen, in the presence of the a27 clone, *hlb* and *hla* transcripts are greatly increased and those of *spa* are completely eliminated.

The observed effects on target gene transcripts could reflect either induction/repression of transcription or stabilization/destabilization of the transcripts. To test for *agr*-specific induction/repression, we prepared and analyzed gene fusions in which the *hla* and *spa* promoters were transcriptionally fused to the staphylococcal plasmid pI258 β-lactamase structural gene, using the transcriptional fusion vector, pSA3800. The fusions were introduced into *agr*$^+$ (RN6390B) and *agr*$^-$ (ISP546) strains and the plasmid-containing derivatives were grown in liquid cultures and

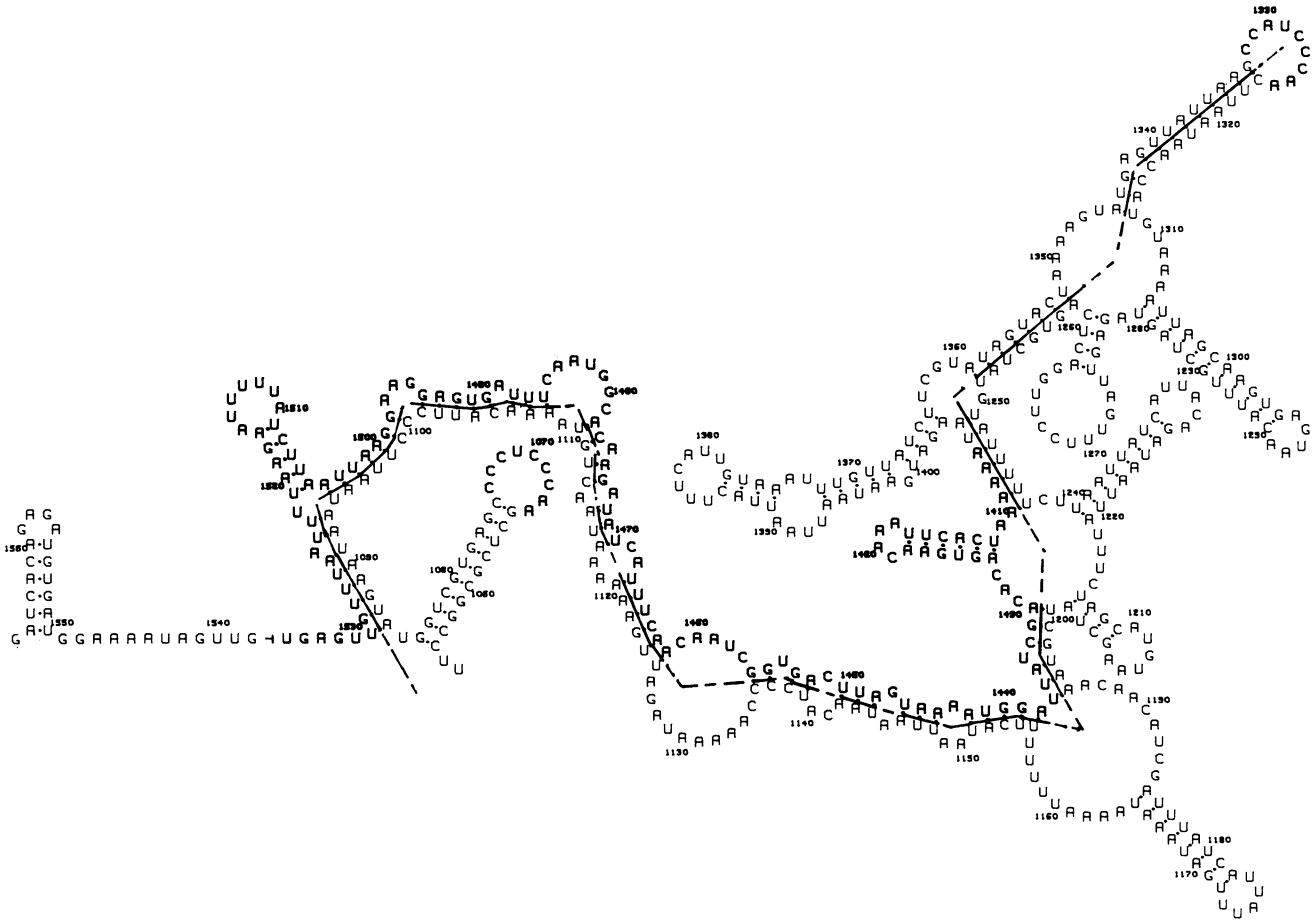

Fig. 3. Predicted secondary structure of RNAIII. The secondary structure was predicted by the Zuker–Stiegler program (Zuker and Stiegler, 1981) and drawn on a Hewlett-Packard plotter with software kindly provided by Dr Fred Kramer. The *pre-hld-hld* ORF is shown in bold type as are the two homologous loops described in the text.

monitored for β-lactamase production as a function of growth. Both fusions demonstrated *agr*-dependent expression of β-lactamase activity similar to that of the native exoproteins, as shown in Figure 5. Thus the *hla*–β-lactamase fusion showed post-exponential induction of β-lactamase activity in the agr^+ host strain but not in the agr^-, and the reverse was true for the *spa*–*bla* fusion. Results similar to those with *hla* were obtained with *tst*–*bla* and *hlb*–*bla* fusions (not shown). These results indicate that *agr* acts by regulating transcription rather than by affecting mRNA stability. The fusion point for the *hla* fusion is at the start point for transcription, which rules out the possibility that RNAIII acts on the *hla* mRNA, for example, by blocking attenuation.

How does RNAIII act?

Several possibilities can be envisioned: (i) one or more translation products are the effectors; (ii) RNAIII is itself the effector; (iii) RNAIII is the effector but one or more RNAIII open reading frames (ORFs) are involved, acting either through their translation products or by the effects of their translation on RNAIII secondary structure. We have approached this question by the construction and analysis of subclones with deletions affecting the RNAIII region and by site-directed mutagenesis to inactivate reading frames.

In Figure 1B is presented a map of the a27 fragment [nucleotides (nt) 1–1566, at top], showing the extent of RNAIII (1566–1058), selected restriction sites, ORFs and the extents of several subclones and deletions.

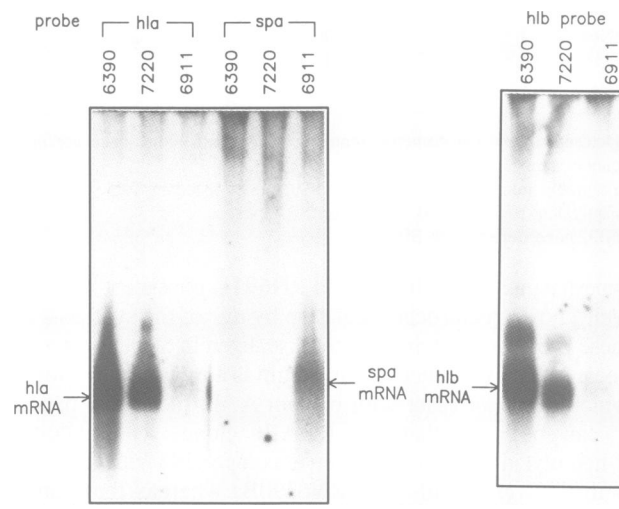

Fig. 4. Effect of RNAIII on target gene transcription. Cultures were grown in CY broth at 37°C with vigorous aeration. Samples were removed in post-exponential phase (5×10^9 c.f.u./ml) and RNA prepared by the Kornblum method (Kornblum *et al.*, 1988) was separated on 1% agarose–formaldehyde and analyzed by Northern blot hybridization (Thomas, 1980) with probes specific for *hla*, *hlb* and *spa* as indicated. Lane 1 contains RN6390B (wt, agr^+) RNA; lane 2 contains RN7220 (*agr*-null with cloned RNAIII determinant) RNA; and lane 3 contains RN6911 (*agr*-null) RNA. The doublet seen with the *hlb* probe could represent processing of the transcript or two independent start points.

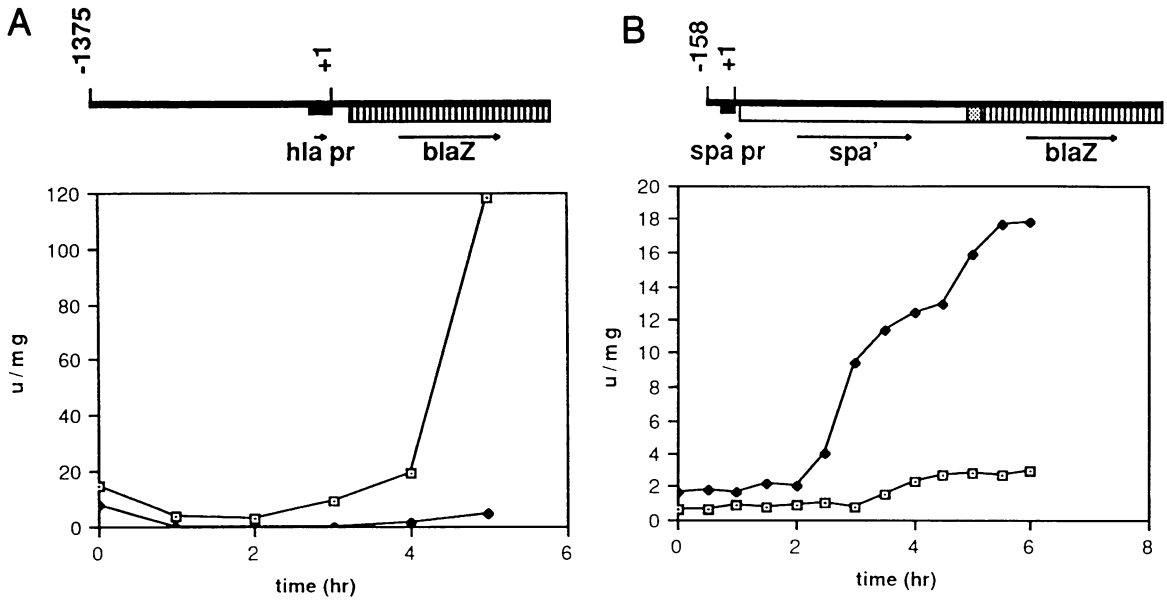

Fig. 5. *agr*-sensitivity of exoprotein promoter–*blaZ* fusions. pSA3800 derivatives containing exoprotein gene promoters fused to *blaZ* were introduced into RN6390B (*agr*$^+$) and ISP546 (*agr*$^-$). The resulting strains were grown in CY broth, starting at 2 × 10^8 organisms per ml, and grown with shaking at 37°C. Samples were removed at the indicated times and assayed for β-lactamase by the nitrocefin method. Each gene fusion is diagrammed above the corresponding graph of β-lactamase activity. (**A**) α-hemolysin; (**B**) protein A.

The 135 nt a41 segment specifying the 3′ end of the RNAIII molecule induced *hla* and *hlb*, albeit rather weakly. More extensive 3′ subclones, such as a39 and a28, activated these two genes more strongly, in proportion to their length, reaching maximum activity at a length of 422 nt (the *agr*-a28 fragment). The 211 nt a29 segment specifying the 5′ end also induced *hla* and *hlb* transcription, leading to the conclusion that non-overlapping segments of the RNAIII molecule have similar regulatory activities. This similarity could represent overlapping function, possibly owing to homologies between the 5′ and 3′ halves of the molecule [see, for example, the two homologous loops (1323–1333 and 1065–1075) shown in bold type in Figure 3]. This possibility is under study. The situation for *spa* repression was somewhat different: although the non-overlapping 3′ and 5′ subclones both had activity, in this case the 135 nt 3′ subclone (a41) was as active as the intact RNAIII molecule, whereas the 5′ subclone repressed *spa* only weakly. These results are shown in Figure 6 and summarized in Figure 1B.

We have used site-directed mutagenesis to test for the possible involvement of RNAIII-encoded peptides or the translation of RNAIII ORFs in exoprotein regulation. We have inactivated the *hld* reading frame by the in-frame deletion of 13 amino acids, using a sequence-skipping primer to delete nucleotides 1466–1428. The resulting clone, a27Δ8, which lacks δ-hemolysin activity, showed the same exoprotein regulation as the parental a27 clone. A similar result was obtained by Janzon and Arvidson who introduced a nonsense mutation at *hld* codon 3 (Janzon and Arvidson, 1990). We have also inactivated the 19 residue ORF (pp19, Figure 1B) by replacing the putative start codon (TTG) at position 1173 with a stop codon (TAG). The mutant RNAIII had the same activity as the wild-type RNA (not shown).

Given the transcriptional activating activity of the 3′ a41 clone, this latter result rules out any absolute requirement for translation of RNAIII in the regulation of exoprotein gene transcription. Although it is clear that RNAIII itself rather

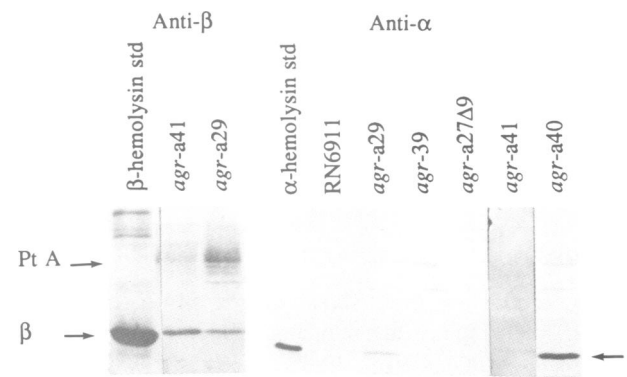

Fig. 6. Western immunoblot analysis of exoprotein production by RNAIII subclones. See Figure 2 legend for details.

than any translation product is the *agr*-determined effector of exoprotein gene regulation, the possibility that translation of the 21-codon ORF (pp21, Figure 1B) or the 5′ extension of *hld* (*pre*, Figure 1B) are involved in the response has not been ruled out. Experiments to inactivate translation of these two regions are in progress.

RNAIII also affects translation

We have observed that the 3′ subclones, including a28, a39 and a41, all stimulated β-hemolysin but not α-hemolysin activity as seen in sheep blood agar (SBA) and by Western immunoblotting (Table I). Additionally, no trace of α-hemolysin antigen could be detected in the cytoplasm of strains containing any of these clones (not shown). This suggests that either the *hla* mRNA is not translated in these strains, or its protein product is degraded. We have analyzed a mixed culture containing equal quantities of *agr* wild-type and *agr*-a41 strains. The same amount of α-hemolysin antigen was produced by the mixed culture as by the *agr* wild-type strain alone (not shown), ruling out degradation

as a possibility. Therefore, it appears that the a41-induced *hla* transcript is not translated. By contrast, α-hemolysin was readily detectable in the supernatant of a strain containing the 5′ subclone, a29 (Table I). This suggests that *hla* mRNA is untranslatable in the absence of the 5′ region of RNAIII, and therefore that RNAIII regulates *hla* transcription and translation independently. The largest 3′ subclone, *agr*-a28 (Figure 1B), lacks only nucleotides 1566–1472 of the RNAIII sequence, suggesting that this 95 nt region is necessary for *hla* translation. As we have not yet subcloned this 95 nt segment on its own, we cannot say whether it is sufficient for α-hemolysin translation. The apparent requirement for RNAIII for α-hemolysin translation would explain the discrepancy between *hla* transcription and α-hemolysin activity seen in the *agr*-null strain, RN6911 (compare Figures 2 and 4). This strain contains equivalent low levels of *hla* and *hlb* transcripts (Figure 4) but produces only β-hemolysin.

Additional evidence regarding the role of RNAIII in translation was provided by observations with the a27Δ9 clone, which is missing the final 5 nt of RNAIII. This clone stimulates transcription of *hla* and *hlb* at least as well as does a40, but neither α-hemolysin nor δ-hemolysin (which is encoded in RNAIII) is translated, and further, *spa* transcription is not repressed. Computer analysis predicts that this molecule will fold very differently from the wild-type RNAIII and will have a complex pseudoknot in the *hld* region. This supports the idea that the configuration of RNAIII is critical not only for δ-hemolysin translation (which is not surprising) but also for α-hemolysin translation. These types of observation suggest that there may be two or more forms of RNAIII with different activities *in vivo*.

Discussion

In this report, we have shown that a 514 nt transcript, RNAIII, can replace the regulatory function of the entire *agr* locus. When cloned under control of the inducible staphylococcal β-lactamase promoter, the induced RNAIII restored both the positive and negative regulatory functions of the *agr* system in a host strain in which the chromosomal *agr* locus has been deleted. These results clearly established that RNAIII is the *agr*-specific effector of exoprotein gene regulation in *S.aureus* and prompted a series of studies to analyze the molecule genetically. These studies, focusing on the up-regulated target genes, *hla* and *hlb*, and on the down-regulated *spa*, revealed that RNAIII acts primarily at the level of target gene transcription and, secondarily, in some cases, at the level of translation. In these cases, transcription and translation are regulated independently.

Our results rule out the possibility that any translation product of RNAIII is the exoprotein gene regulator; they do not rule out the possibility that the translation process could play a role by altering the secondary structure of RNAIII. In this connection, the translation defect seen with the a27Δ9 clone (Figure 1B) suggests that wild-type RNAIII may require a conformational change to activate its function, a change that is blocked by the secondary structural alteration caused by the Δ9 deletion.

The key unresolved question is how does RNAIII regulate transcription? Most known regulatory RNAs interact with target transcripts, blocking translation (Simons and Kleckner, 1988) or causing transcriptional attenuation (Novick *et al.*, 1989b). This possibility is ruled out by the *hla–bla* fusion, which showed full *agr* sensitivity but contained no part of the *hla* transcript. Similar results have been obtained by D.Sullivan and M.Kehoe (personal communication).

One possible mechanism is via accessory protein factors. RNAIII could either control the synthesis (translation?) of such factors or could interact with them directly, perhaps by forming a multi-functional nucleoprotein complex. The existence of such factors is supported by several types of evidence. Treatment of crude cell lysates with SDS caused a substantial decrease in the sedimentation rate of the RNAIII molecule in neutral sucrose gradients (J.Kornblum and R.P.Novick, unpublished data), implying the existence of an RNA–protein complex. The existence of accessory factors is suggested also by the isolation of globally exoprotein-defective (XOP^-) mutations unlinked to *agr* (Ross, 1989; Cheung *et al.*, 1992; Smeltzer *et al.*, 1993). Additionally, we have recently demonstrated that a second activating signal is required for the post-exponential phase up-regulation of exoprotein genes (Vandenesch *et al.*, 1991). Finally, we have observed that at inhibitory concentrations, antibiotics such as erythromycin (Em) mimic the effects of RNAIII on target gene transcription. Up-regulated genes are induced by Em and down-regulated genes are repressed (unpublished data). These results could indicate that the continued synthesis of labile proteins is needed to maintain the basal regulatory state and that these proteins are antagonized by RNAIII.

It is also possible that RNAIII could interact directly with regulatory sequences in target DNA. The only known

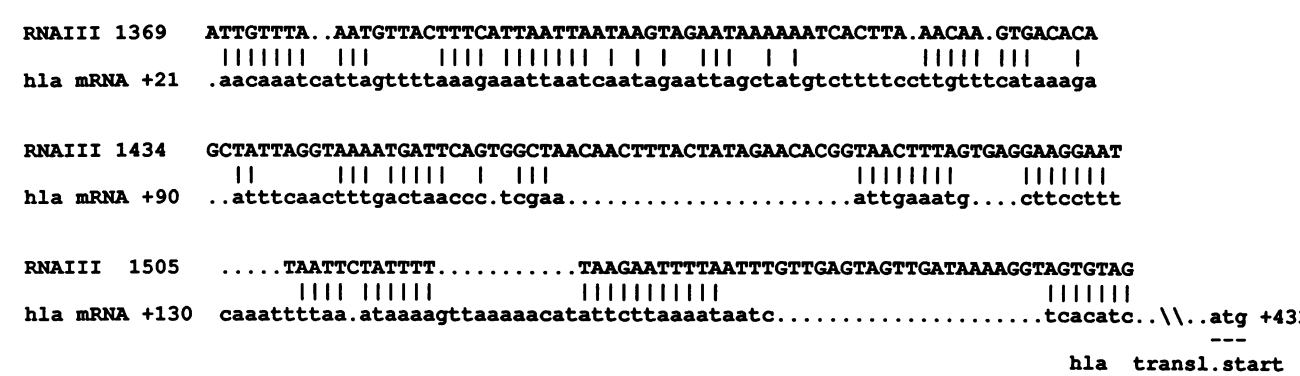

Fig. 7. Complementarity between RNAIII and 5′ sequences in *hla* transcripts. Complementary regions were identified by computer and the alignments completed manually. Numbering of RNAIII sequences corresponds to that in Figure 3. Numbering of *hla* sequences corresponds to the known transcriptional start point for the transcript (D.Sullivan and M.Kehoe, personal communication).

means by which an RNA species could directly affect DNA function is by forming a triple-stranded structure involving a polypurine/polypyrimidine tract (Miller and Sobell, 1966). As neither the known target genes nor RNAIII possess such a sequence, this possibility seems unlikely. The possibility of an RNA–DNA interaction by some other means cannot be ruled out.

With respect to translation, our results have established that the 5′ end of RNAIII is required for α-hemolysin translation. We have observed a striking complementarity between the 5′ end of RNAIII and the 5′ end of the untranslated *hla* leader, as shown in Figure 7. This complementarity could be related to the apparent requirement of 5′ RNAIII sequences for translation of the *hla* mRNA.

A puzzling aspect of the 3′ end subclones is that they appear to eliminate the production of many exoproteins in addition to α-hemolysin (unpublished data). However, the leader regions of other known *agr*-up-regulated exoprotein genes do not show significant sequence complementarity with the 5′ end of RNAIII. Therefore, it is unlikely that RNAIII regulates these proteins by interacting with the 5′ leaders of their mRNAs, as it may do with the *hla* leader.

Why should staphylococci have evolved an RNA-based global regulator for accessory exoprotein genes when other organisms use regulatory proteins for similar purposes? There is a sound evolutionary rationale for other regulatory RNAs; these are generally antisense RNAs that inhibit the function of target RNAs by complementary base pairing; such transcripts could evolve by the simple expedient of developing an antisense promoter within the region of the target RNA. No such simple rationale could account for RNAIII and one must consider more complicated possibilities such as a multifunctional ribonucleoprotein complex whose function would be sensitive to the complicated signals generated *in vivo* or during growth phase transitions *in vitro*.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table II. JM109 was the *Escherichia coli* host strain used throughout. RN6390B is an *agr*$^+$ variant of RN450, our standard laboratory strain, which has an *agr* defect of unknown origin. RN6911 is a derivative of RN6390B in which the 3.4 kb region between the *Cla*I (1187) and *Hin*dIII (446) sites has been replaced by a 3 kb fragment containing the cloned *S.aureus tetM* marker (Nesin *et al.*, 1990) (see below). At least 135 nt beyond the *Cla*I site are also missing in RN6911, presumably owing to a spontaneous deletion. RN7206 (β-null) is a derivative of RN6911 lysogenic for *S.aureus* phage φ13, whose attachment site is within the β-hemolysin structural gene (Coleman *et al.*, 1986). RN7270 (α-null) is a derivative of RN6911 in which *hla* has been inactivated by insertion of the *ermC* gene from pE194 [kindly provided by T.Foster (O'Reilly *et al.*, 1986)]. RN4220 is a nitrosoguanidine-induced mutant of RN450 capable of accepting *E.coli* DNA (Kreiswirth *et al.*, 1983). pE194 is a naturally occurring staphylococcal erythromycin

Table II. Bacterial strains and plasmids

	Description	Reference or source
Strains		
Wood46	naturally occurring α-hemolysin producer	ATCC
ISP546	RN6390B *agrA316* (Tn*551*)	Mallonee *et al.* (1982)
RN4220	restriction$^-$ mutant of RN450, partially *agr*$^-$	Kreiswirth *et al.* (1983)
RN6390B	NTCC8325 cured of three prophages	Peng *et al.* (1988)
RN6911	RN6390B Δ*agr*1057–4546::*tetM*	This work
RN7206	RN6911 (φ13)	This work
RN7220	RN6911 (pRN6735)	This work
RN7266	RN7271 (pRN6735)	This work
RN7270	RN6911 *hla*::ermC	This work, O'Reilly *et al.* (1986)
RN7271	RN6911 (φ13) *hla*::ermC	This work
NTCC8325	naturally occurring *agr*$^+$	NTCC (UK)
Plasmids		
pSK265	pC194::pUC19 polylinker at *Hin*dIII site	Ranellin *et al.* (1985)
pI524	naturally occurring β-lactamase plasmid	Novick (1963)
pSA3800	*bla* fusion shuttle vector derived from pWN2018 (Wang *et al.*, 1987)	S.Iordanescu[a]
pRN5543	pSK265Δ *Hin*dIII site	This work
pRN5548	pRN5543::p-*bla bla* promoter vector	This work
pRN6676	pSA3800::p-*tst*	This work
pRN6704	pSA3800::p-*hlb*	This work
pRN6725	pRN5543::P-bla *blaZ′* promoter vector	This work
pRN6735	pRN6725::*agr*-a27	This work
pRN6741	pRN6725::*agr*-a28	This work
pRN6743	pRN6725::*agr*-a29	This work
pRN6744	pTN6725::*agr*-a30	This work
pRN6788	pSA3800::p-*spa*	This work
pRN6819	pRN6725::*agr*-a27Δ9	This work
pRN6820	pRN6725::*agr*-a39	This work
pRN6827	pSA3800::p-*hla*	This work
pRN6828	pRN6725::*agr*-a27Δ8	This work
pRN6848	pRN5548::*agr*-a40	This work
pRN6851	pRN5548::*agr*-a41	This work
pRN6852	pRN5548::*agr*-a42	This work
pRn6873	pRN5548::*agr*-a40.2 nonsense mutation in pp19	This work

[a]Personal communication.

resistance plasmid (Horinouchi and Weisblum, 1982). pWN2018 is a derivative of pC194 containing the ColE1 replication origin and a promoterless derivative of the pI258 β-lactamase structural gene preceded by the pUC18 polylinker (Wang *et al.*, 1987). pSA3800 is a derivative of pWN2018 with stop codons in all three reading frames between the polylinker and the *blaZ* start (constructed and kindly provided by S.Iordanescu).

Media and growth conditions

E.coli cultures were grown in L broth. *S.aureus* cultures were grown in CY broth (Novick and Brodsky, 1972) supplemented with glycerol phosphate (0.1 M) or on GL agar (Novick and Brodsky, 1972) with antibiotics as indicated. Tetracycline (Tc), chloramphenicol (Cm) and erythromycin (Em) were each used at 5 μg/ml. Liquid cultures were shaken at 37°C and monitored turbidometrically with a Klett–Summerson photoelectric colorimeter read at 540 nm. A Klett value of 343 for *S.aureus* corresponds to 1 mg dry wt/ml. Transduction was performed with phages φ11M15 or 80α (Novick,1967). Transformation of *E.coli* was performed using frozen competent JM109 cells as described (Sambrook *et al.*, 1989). Protoplast transformation of *S.aureus* was performed as described by Chang and Cohen (1979) using lysostaphin (Applied Microbiology, Inc., New York) in place of lysozyme.

Nucleic acids

Plasmid DNA was extracted as previously described (Novick *et al.*, 1979). DNA sequencing was by the dideoxynucleotide method (Sanger *et al.*, 1977). Enzymes were purchased from Boehringer Mannheim, New England Biolabs or Perkin Elmer–Cetus. RNA was prepared by the method of Kornblum *et al.* (1988) or by the method of Chirgwin *et al.* (1979). Northern blot hybridization was performed by the method of Thomas (1980), using the following probes:

hla–ClaI–C (1375–1909) (Gray and Kehoe, 1984), nick-translated;
hlb–RsaI–A (1–2186) (Projan *et al.*, 1989), and unpublished data, nick-translated;
RnaIII, PCR product, nt 1320–1566 (Figure 1B);
primers: 5′-ATGATCACAGAGATGTGA
5′-CTGAGTCCTAGGAAACTAACTC
spa, PCR product, nt 776–1160 (Uhlen *et al.*, 1984);
primers: 5′-ATCTGGTGGCGTAACAC
5′-CAGCTTTCGGTGCTTG

Nick-translation was performed by the method of Rigby *et al.* (1977). S1 nuclease mapping was performed by the method of Berk and Sharp (1977). T7 polymerase *in vitro* run-off transcripts of 2800 and 379 nt, prepared from a clone of *agr*-a27 in M13, were used as size standards for Northern blots. The polymerase chain reaction (PCR) was performed with a Perkin Elmer–Cetus thermocycler using Taq polymerase. Reactions (50 μl) contained 200 ng each primer, 1–20 pg template, 20 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.05% Tween 20, 100 μg/ml gelatin, 2 U Taq polymerase, four dNTPs, each at 200 μM. Reactions were run for 30 cycles: 30 s at 94°C, 30 s at 50°C and 1 min at 72°C. PCR products were first cloned to PCR-1000 (Invitrogen) and then to a staphylococcal vector. For radioactive labeling, dATP was reduced to 1.5 μM and 20–50 μCi [α-^{32}P]dATP were added. Unincorporated counts were removed by gel filtration (Centricon 30) and radioactivity determined by scintillation counting in Ultrafluor (National Diagnostics). By this method, specific activities in excess of 10^9 d.p.m./μg were obtained.

Constructions

pRN5543 and derivatives. The *Hin*dIII site in pSK265 (Ranellin *et al.*, 1985) adjacent to the *Eco*RI site of the pUC19 polylinker was filled in with polymerase I (Klenow fragment) and the resulting plasmid designated pRN5543. pRN6725 was constructed by ligating an 839 nt *Hin*dIII–*Xba*I fragment, from *S.aureus* plasmid pI258, containing the β-lactamase promoter and part of the *blaZ* gene (Wang and Novick, 1987) to the polylinker region of pRN5543. pRN5548 was constructed by cloning into *Hin*dIII-digested pRN5543 a 164 nt PCR product prepared using pI258 as a template and primers 5′-AAGCTTACTATAGCCATT-3′ and 5′-AAGCTTAGCTCT-CTAGCTACCTAGCAATAAACCCTCC-3′ homologous to sequences flanking the β-lactamase promoter (positions −105 to −88 and +24 to +35, respectively) in the numbering system of Wang and Novick (1987). The 3′ primer also contains codons in all three reading frames. With either of these vectors, genes cloned at the polylinker site are controlled by the β-lactamase promoter. This promoter is indifferent to *agr* (unpublished data) and, in the absence of the *bla* represssor, is expressed constitutively. In the presence of the repressor, it is expressed at a low basal level and can be induced ~30-fold by β-lactamase inducers such as methicillin (Novick, 1962) or carboxyphenylbenzoyl aminopenicillanic acid (CBAP) (unpublished data). Derivatives of vectors pRN6725 and pRN5548 containing pUC18::*agr*-a0 (Peng *et al.*, 1988) subfragments (designated by 'a' plus a number in order of isolation) a27, a28, a29, a30, a39, a40 and a41, were prepared by ligating gel-purified restriction fragments, as indicated in Figure 1, to appropriately digested vector preparations and transforming RN7497 protoplasts with selection for the vector Cm^r marker. The a42 fragment was prepared as a PCR product using *agr*-a0 as template and primers 5′-GCTGCAGCTAAATCGTATAATGACAG and 5′-GTTAACTGAC-TTTATTATG, homologous to *agr* positions 1737–1755 and 4532–4550, respectively. This PCR product was cloned first into pBluescript, then to pRN5548. The a27Δ9 derivative was prepared by eliminating a 300 nt *Fnu*dII fragment from pRN6735 (the a27 derivative of pRN6725).

agr-a27Δ8. A commercially obtained 30 nt primer matching the sequences from 1413–1427 and 1467–1481 and omitting the 39 nt from 1428–1466 (see Figures 1 and 3) was used to prime complementary strand synthesis on an M13mp18 derivative containing the cloned *agr*-a21 fragment [*Pvu*II(330)–*Pvu*II(4150)], using the method of Kunkel *et al.* (1978). The 1197 nt *Mbo*I–*Bam*HI fragment containing the deletion was then cloned into pRN6725 and the deletion confirmed by sequencing.

agr-a40.2. Synthetic oligonucleotides, 5′-CATCGATTTATCATTATA-GATAAATAAAAT (containing a substitution of TAG for TTG at 1185–1183) and 5′-CGAATTCAGCTCCGGTA, corresponding to positions 1186–1162 and 824–840, respectively, were used to prime the PCR reaction using the *agr*-a27 fragment as template. The resulting PCR product was cloned to PCR-1000 (Invitrogen) and then used to reconstruct the intact *malII* determinant (824–1566, corresponding to *agr*-a40) in pRN5548. The *malII* region of the resulting plasmid (pRN6873) was sequenced to confirm the induced mutation.

RN6911. The pUC18::*agr*-a0 clone (Peng *et al.*, 1988) was digested with *Cla*I and *Hpa*I and the larger (5 kb) fragment gel purified and cloned into a 3 kb *Cla*I–*Sma*I fragment containing the *S.aureus tetM* gene (Nesin *et al.*, 1990). Plasmid DNA from *E.coli* containing this clone was used to transform *S.aureus* RN4220 *agrA316* (Tn*551*) with selection for Tc^r, resulting in a Campbell insertion of the entire plasmid. The resident copy of the *agr* locus was eliminated by a transductional outcross and the resulting construct, RN6911, confirmed by Southern blot hybridization. Unexpectedly, *agr* sequences to the right of the *Cla*I (1187) site (at least 135 nt) were also missing, presumably owing to a deletion in this region. RN6911 is referred to as '*agr*-null'. RN6911, like all other agr^- mutants, produces a trace of β-hemolysin but no detectable α-hemolysin.

bla fusions. Transcriptional fusions using a promoterless derivative of pI258 *blaZ* as the reporter gene were constructed by ligating promoter-containing fragments (see Figure 5) of the cloned *hla*, *spa*, *hlb* and *tst* determinants to suitably digested pSA3800. Location of the *spa* promoter was determined by Patel *et al.* (1992); location of the *hla* promoter was determined by D.Sullivan and M.Kehoe (personal communication); locations of the *tst* and *hlb* promoters were inferred from the mRNA start sites determined by the method of Berk and Sharp (1977).

Exoprotein analysis

Culture supernatants were dialyzed, concentrated 10-fold by vacuum evaporation and separated by PAGE according to the Laemmli (1970) procedure. Gels were analyzed by Western immunoblotting as described by Blake *et al.* (1984).

β-Lactamase was assayed colorimetrically on samples of whole culture using nitrocefin as substrate (O'Callaghan *et al.*, 1972). One unit of activity corresponds to 1 μmol/h. Activities measured with nitrocefin were ~12% of those obtained by assays using penicillin G as substrate.

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